STUDIES ON A VIRUS CAUSING MOSAIC DISEASE ON SQUASH (*Cucurbita pepo* L.)

DISSERTATION

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By

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In the Memory of
My
Late Grandfather

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Certificate

This is to certify that the dissertation entitled "Studies on a virus causing mosaic disease on Squash (*Cucurbita pepo* L.)" submitted in partial fulfilment of the requirements for the award of the degree of Master of Philosophy in Botany (Plant Virology) is a faithful record of the bonafide research work carried out at Aligarh Muslim University, Aligarh by **Mr. Abid Rafiq** under my guidance and supervision and that no part of it has been submitted for any other degree or diploma.

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Chapter -1
Introduction

INTRODUCTION

The existence of life is not possible without the existence of plants because every aspect of life is tightened with the plants. For the existence of iife at any level, there are three great needs of life, food, clothing and shelter. Out of these food is of prime importance. Plants contribute huge quantity of food and of great variety e.g. cereals, legumes, fruits and vegetables etc. for the millions of creatures comprising the animal kingdom including human being.

Vegetables do not only adorn the table but also enrich health of man. Regular use of vegetables supplies many of the most essential health building and protecting substances such as vitamins and minerals which are wanting in other food materials. Vegetables if taken fresh are more vigorating than cooked. They gives us Himalayan health and resplendent redness which is unfailing symbol of health, life, cheerfulness and ecstasy. Those who use vegetables less or those who are not in position to afford them suffer from mineral deficiency diseases. Vegetables play an important role in neutralizing the acids produced during the course of digestion of meat, cheese and other fatty foods and they provide roughage which promotes digestion.

Vegetables hold a high potential for combating the food storage as their yield per unit area is more than five times of any cereal crops. India grows a large variety of vegetables belonging to tropical,

subtropical and temperate groups. In India vegetables constitute hardly 8-10 percent of the total food intake which is distressingly low as compared to 45% in Japan. Even in countries like America where annual consumption of vegetables per capita is almost five times that of an average Indian. Their availability in our country is only 1.3 ounces per adult per day while on the other hand balanced diet requires 10 ounces of vegetables per adult per day.

Among vegetables cucurbits are of prime importance in day to day life. The cucurbits represented by about 118 genera and 825 species of family cucurbitaceae are widely distributed in tropics and subtropics and a few species also occurs in the temperate regions. At least 35 species, belonging to 16 genera, are widely used as fruits and/or vegetables, which include bitter gourd, bottle gourd, cucumber, musk melon, pumpkin, ridge gourd, sponge gourd and water melon (Table 1). Cucurbits are important source of vitamins, minerals, fibres, oil, medicines, and production of some useful articles (Kirtikar and Basu, 1983; Nayar and Singh, 1998).

Cucurbits grow well in alluvial and sandy soils even in arid regions and coastal saline areas (Guha and Sen, 1998). In the Indo-gangatic plains of Northern India, cucurbits although susceptible to cold are sown in poly bags at the beginning of winter and transplanted to the river beds under mulches during the early spring. In West Bengal, rainy season cucurbits are sown in June-July and in South and Central India in

April-May. Thus cucurbits are grown in the country almost throughout the year.

Viral diseases are a major constraint in the production of cucurbits in India and also limit the economic empowerment of resource poor farmers, who are the main growers of these crops mostly on the marginal lands. The cucurbits are reported to be naturally infected by more than 10 viruses. The most common viruses affecting cucurbits are cucumber mosaic virus (CMV), cucumber green mottle mosaic virus (CGMMV), water melon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV) and tomato spotted wilt virus (TSWM) (Raychaudhari and Varma, 1978; Vani, 1987; Jain *et al.*, 1998; Varma and Giri, 1998; Mandal *et al.*, 2003). These viruses cause symptoms like mosaic, mottling, vein clearing, leaf distortion and leathery appearance of older leaves. Infected plants remain stunted with poor fruit setting, which results in reduction of yields.

The plant for present study is *Cucurbita pepo* L. of family cucurbitaceae. The plant also known as vegetable marrow, field pumpkin etc. Local names are Bilaiti Kaddu, Chappan Kaddu, Kumara yellow Bhopala, etc. *C. pepo* is favourate and popular vegetable. It is used green or matured, when its colour is changed to yellow or red. It is of American origin. The center of origin is Gutemala and Central Mexico or Colombia. The plant is cultivated through out India.

Fruits can be stored for months under proper conditions of temperature and humidity. Fruits of pumpkins are extensively used as vegetables. They are used as fresh vegetables, processed food and stock feed. They are sweetish when fully mature and can be used in preparing sweets and jams. They can also be candied or fermented to give a beverage.

The fresh seeds are antihelminthic and useful as *tuenicide*. Analysis of edible portions of fruits contain moisture 94.8%, protein 0.5%, fats 0.1%, carbohydrates 4.3% mineral matter 0.3%, Ca 0.01%, P 0.03%, Fe 0.6%, Vitamin C 18mg/100gm.

Like other cucurbits *C. pepo* L. is also attacked by several viruses viz. Cucumber mosaic virus (CMV), Water melon mosaic virus (WMV), Zucchini yellow mosaic virus (ZYMV) and Squash mosaic virus (SqMV).

The present investigations embodies some preliminary studies on the virus causing mosaic disease on squash (*C. pepo* L.) with following objectives.

(i) Host range

(ii) Transmission

(iii) Symptomatology

(iv) Biophysical properties (DEP, TIP and Longevity in vitro).

(v) Effect of various buffers at different pH levels and molarity on the infectivity of virus.

Table 1: Cucurbits cultivated in India.

Common English name	Hindi name	Botanical name
Ash gourd	Petha	*Benincasa hispida*
Bitter gourd	Karela	*Momordica charantia*
Bottle gourd	Lauki or Ghiya	*Lagenaria siceraria*
Cucumber	Khira	*Cucumis sativus*
Indian squash (Round melon)	Tinda	*Praecitrullus fistulasus*
Ivy gourd or scarlet	Kundru	*Coccinia cordifolia*
Long or serpent melon	Kakri	*Cucumis melo* var. utilissimus
Musk melon	Kharbuza	*Cucumis melo*
Pointed gourd	Parwal	*Trichosanthes dioica*
Pumpkin	Sitaphal	*Cucurbita moschata*
Ridge gourd	Kali tori	*Luffa acutangula*
Snake gourd	Chichinda	*Trichosanthes cucumerina*
Snap melon	Phoot	*Cucumis melo* var. momordica
Sponge gourd	Ghia tori	*Luffa cylindrica*
Squash	Chappan kaddu	*Cucurbita pepo*
Squash or winter squash	Kashiphal	*Cucurbita maxima*
Sweet gourd of Assam	Kheksa	*Momordica cochinchinsis*
	Meetha karela	*Cyclanthera pedata*

Chapter -2
Review of Literature

REVIEW OF LITERATURE

Cucurbits are known to be naturally infected by nearly 50 plant virus species belonging to 14 genera, of these 22 belong to Begomovirus and 6 to Potyvirus (Table 2), (Brunt *et al.*, 1990; Varma & Giri, 1998; Muniyappa *et al.*, 2003; Singh *et al.*, 2001; Avgelis, 1985 and Mandal *et al.*, 2003)

A new strain of vegetable marrow mosaic virus was reported by Naqvi & Mehmood (1975). The infected plants showed mosaic mottling, stunting and reduction in the number of flowers and fruits. The virus was transmitted mechanically by sap inoculation and also by aphids, viz. *Aphis gossypii, Brevicoryne brasicae* and *Myzus persicae.* The host range of virus was restricted to the families Cucurbitaceae and Solanaceae. The longevity *in vitro* was found upto 96h at 20-26°C which lost at 108h. The thermal inactivation point was 65°C and the dilution end point 10^{-5}.

Ghosh *et al.*, (1977) in their studies found flexous rod particles from pumpkin (*C. moschata* Poirs.) using electron microscopy. All these virus isolates differ in their length and breadth. Depending upon their dimensions these isolates were identified as bottle gourd mosaic virus, cucurbit latent virus, cucumber vein yellowing virus, pumpkin mild mosaic virus and cucurbit mosaic virus.

Table 2: World and Indian scenario of plant viruses infecting cucurbits.

Groups	Viruses	Other countries	India
Carlavirus (1)	*Muskmelon vein necrosis*	+	-
Cormovirus (1)	*Melon necrotic spot*	+	-
Closterovirus (2)	*Lettuce infectious yellow virus*	+	-
	Beet Pseudo yellow virus	+	-
Comovirus (1)	*Squash mosaic virus*	+	-
Crinivirus (1)	*Cucumber yellow stunting disorder virus*	+	-
Geminivirus (22)	*Chayote yellow mosaic virus*	+	+
	Cucumber yellow vein virus	+	-
	Cucurbit leaf crumple virus-[Cal]	+	-
	Cucurbita maxima yellow mosaic virus	-	+
	Luffa yellow mosaic virus	+	-
	Melon chlorotic leaf curl virus-[CR]	+	-
	Melon chlorotic leaf curl virus-[GT]	+	-
	Melon leaf curl virus	+	-
	Pumpkin yellow mosaic virus	-	+
	Squash leaf curl virus-[Cairo]	+	-
	Squash leaf curl Israel virus	+	-
	Squash leaf curl China virus	+	-
	Squash leaf curl China virus-[Pum:Coim]	-	+
	Squash leaf curl China virus-[Pum:Del]	-	+
	Squash leaf curl China virus-[Pum:Luc]	-	+
	Squash leaf curl Philippines virus	+	-
	Squash leaf curl Yunnan virus [Y23]	+	-
	Squash mild leaf curl virus-[IV]	+	-
	Squash mild leaf curl virus-[R]	+	-
	Tomato leaf curl New Delhi virus-[Luffa]	-	+
	Watermelon chlorotic stunt virus	+	-
	Watermelon chlorotic stunt virus-[IR]	+	-

Ipomovirus (1)	*Cucumber vein yellowing virus*	+	-
Luteovirus (1)	*Beet western yellow virus*	+	-
Necrovirus (1)	*Tobacco necrosis virus*	+	-
Nepovirus (3)	*Kakari mosaic virus*	-	+
	Muskmelon ring spot virus	-	+
	Tobacco ring spot virus	+	-
Potyvirus (6)	*Bean yellow mosaic virus*	+	-
	Moroccan watermelon mosaic virus	+	-
	Watermelon mosaic virus-1	+	+
	Watermelon mosaic virus-2	+	+
	Zucchini yellow fleck virus	+	-
	Zucchini yellow mosaic virus	+	+
Tobamovirus (2)	*Cucumber green mottle mosaic virus*	+	+
	Kyuri green mottle mosaic virus	+	-
Tospovirus (2)	*Tomato spotted wilt virus*	+	-
	Watermelon bud necrosis virus	-	+
Tymovirus (1)	*Melon rugose mosaic virus*	+	-
Unknown virus	*Cucumber yellow virus*	+	-
	Cucurbit latent curl virus	+	+
	Pumpkin enation mosaic virus	-	+
	Pumpkin mild mosaic virus	-	+

Source: Brunt *et al.*, 1990; Varma and Giri, 1998; Muniyappa *et al.*, 2003; Singh *et al.*, 2001, Avgelis, 1985, Mandal *et al.*, 2003.

Leaf curl disease of squash was reported by Benigno (1978). The disease was seed borne and transmitted by *B.tabaci* in a semi persistent manner but not mechanically by sap. Symptoms include severe stunting, upward leaf curling and reduction in the size of leaves. Vein on both leaf surfaces become prominent and interveinal areas corrugated with slight chlorosis. Enation occurred later on the lower surface.

Effect of some rod shaped cucurbit viruses on total chlorophyll content of the leaves of *C.moschata* Poirs. plant at different stage of pathogenesis was reported by Ghosh (1978). The 9 virus isolate altered the chlorophyll content in inoculated squash leaves in different ways, 5 types of alteration being distinguished.

Effect of some pesticide on the incidence of a virus disease of pumpkin (*C.moschata* Poirs.) was reported by Roy and Mukhopadhyay (1979). When 3 insecticides were applied at transplanting and 40 days later Disyston 5G (disulfoton) proved superior in delaying symptoms of water melon mosaic virus 1 and improving yields.

Effect of plant population on the incidence of mosaic disease and productivity of vegetable marrow (*C.pepo* L.) was reported by Eegla and Badr (1979). Infection was caused by CMV and WMV-2 in the spring and mainly WMV-2 summer. An increase in plant population when the distance between them being reduced from 50 to 20 cm was noticed. There was decreased infection in the summer and increased yield.

Incidence, economic importance and prevention of WMV-2 in squash (*C.pepo*) fields in Jordon was reported by Mansour and Al-Musa (1982). A survey showed that this is the most prevalent virus on squash. In spring crops infection occurred early and increased rapidly, whereas in autumn crops it occurred later and increased at slower rate. Infection reduced yield by 50-100%. Disease spread was reduced by weekly sprays of stylet oil.

Occurrence of viral diseases of melons and squash in the San Fransisco region was reported by Chaudhary and Lin (1982). Water melon mosaic virus-1 was detected serologically by immunodiffusion on 3 melon and 1 squash specimens from 3 fields. Cucumber green mottle mosaic virus was possibly present on 2 of the melon specimens.

Relationship of pumpkin mosaic virus with its aphid vector, *Aphis gossypii* Glov. was reported by Singh (1982a). Pre-acquisition fasting (minimum 10 min. opt-90 min.) was essential for virus transmission to pumpkin, acquisition and transmission occurred in 20 and 10 sec. respectively opt. feeding period were 10-15 and 15-30 min. respectively. Transmission was maximum with 10 aphids/plants. Post acquisition fasting of 2hr. resulted in loss of infectively. In serial transfer with interval between feeding on successive plants of 10 min. or more only the Ist plant was infected, while with interval of 2 and 5 minutes, 3 and 2 plants could be infected respectively but not subsequent ones, indicating that the virus was non persistent. A late and apterous forms transmitted the virus equally efficiently.

Singh (1982b) reported effect of pumpkin mosaic virus infection on the Hill reactions and primary productivity of pumpkin (*C. maxima* Duch) leaves. Production of dry matter was reduced and respiration rate increased in infected leaves compared with healthy ones. Limiting values being noted 60 days after inoculation. The rate of Hill reaction was higher in healthy samples than in infected ones at comparable ages.

Natural virus infection in silvery and non silvery lines of *C. pepo* was reported by Davis and Shifriss (1983) under natural condition. Severity of virus disease was consistently lower in the summer squash silvery leaved breeding cultivar early prolific straight neck (EPS) throughout the season. About 6 weeks after planting, 99% of EPS and 28% of NJ 260 plants showed symptoms of cucumber mosaic virus and clover yellow vein virus. By the end of season, all plants of both lines had developed symptoms, although EPS plants were much more severely effected. Disease related survival was 96% in NJ 260 until the first mid season and at the last reading before frost only 19% of the plants were alive.

Purification, serology and vector relationships of squash leaf curl virus, a white fly transmitted geminivirus was reported by Cohen *et al.*, (1983). A complex of white fly borne disease agent was isolated from field cucurbits in the South Western desert of U.S.A. One component, SLCV alone caused severe stunting and leaf curl symptoms on leaves of all cultivars of *C. maxima, C. moschata* and *C. pepo* that were tested,

and a green mosaic or leaf distortion of *Phaseolus vulgaris*. The virus was purified by differential centrifugation after clarification of leaf extracts with chloroform. Virus yields reached 130µg /100gm of plant material. The $A_{260/280}$ ratio was 1.5. Infectivity assayed by *B. tabaci* fed through membranes, was associated with the occurrence of virus particles (22x38nm) which, however, were not completely separated from monomers trimers and tetramers. SLCV is circulative in *B. tabaci* which has a relatively long latent period. A high frequency of transmission following the latent period is associated with apparent harmful effects of the virus on the vector. SLCV may multiply in the white flies, serological studies showed SLCV is related to cassava latent virus, but not to four other geminiviruses.

Relation of aphids and thrips to the spread of mosaic disease on some *C. pepo* L. varieties at Alexandria, Egypt was reported by Mesbah *et al.*, (1983). The relationship between levels of mosaic virus infection and thrips vector infestation of squash varieties were tabulated.

Singh (1983) reported changes in different phosphorous fraction in watermelon mosaic virus infected pumpkin plant. Total and organic phosphorus levels were higher in virus infected leaves at all stages of growth in comparison to healthy controls. Inorganic phosphorus was low in virus infected plants.

Tiwari and Shukla (1984) reported Peroxidase activity of *C.maxima* Dusch. infected with three strain of watermelon mosaic virus.

Peroxidase activity was higher in infected than in healthy (squash) plants.

Pink and Walkey (1984), reported resistance in Marrow (*C.pepo* L.) to different strains of cucumber mosaic virus. Plants of 3 cultivars were inoculated with 6 CMV structure from different geographical areas which also differ in pathogenicity and virulence Cindrella was highly resistant, Cobham bush green and Gold brush was highly susceptible particularly to all CMV which caused necrotic symptoms.

Purification and serology of squash mosaic virus isolated from watermelon was reported by Lima and Amaral (1985). A virus was isolated from *Citrellus lantalus* plants with severe mosaic and systemic necrosis in irrigated field in Piani, Brazil. It was serologically identified as squash mosaic virus. The purified virus was highly infective. The antiserum reacted specially with the purified virus and sap from infected plants, but not from healthy plants.

Bishnoi *et al.*, (1985) reported mosaic disease of summer squash (*C.pepo* L.) in Haryana. Symptoms include mosaic, vein chlorosis and leaves reduced and becoming filiform, with dark raised areas. Infection occurs at an early stage and the disease which is restricted to the cucurbitaceae, is transmitted by *Aphis gossypii, A. malvoides* and *M. persicae*. Particles were filamentous (700-730 x 15nm) with a longevity *in vitro* at 25-30°C of 24-38 hour, dilution end point of 10^{-3} –10^{-4} and

thermal inactivation point of 45-50°C. The virus is serologically related to watermelon mosaic virus.

Watermelon mosaic virus in pumpkin was reported by Tripathi and Joshi (1985). A sap transmissible virus producing mosaic and leaf distortion symptoms on pumpkins in the Basti area, Uttar Pradesh was efficiently transmitted by *Aphis gossypii, A. craccivora* and *M. persicae*. The host range included members of the chenopodiaceae, compositae, cucurbitaceae and leguminosae. The virus had a dilution end point of 10^{-5}–10^{-6}, a thermal inactivation point of 60–65°C and longevity *in vitro* of 26–27 days at 32–34°C and 42 days at 17–19°C. It resembles a structure of watermelon mosaic virus.

Thourenel *et al.*, (1986) reported occurrence of watermelon mosaic virus 1 in Niger. The virus is newly reported from Tropical Africa on Pumpkin (*C.pepo*) in a field near Arlit, Niger. The disease was not seed transmissible.

First report of Zucchini yellow mosaic viruses in Ohio was reported by Nameth in the year (1986). The virus has recently been identified as ZYMV in Ohio on pumpkins.

Mc Leod *et al.*, (1986) reported ZYMV, a new severe cucurbit disease. Samples from Zucchini, squash and watermelon crops taken during 1985, indicated that the prominent virus causing disease in Western and Northwestern parts of the state was ZYMV which was

responsible for severe losses often approaching 100%. In samples from Central Arkansas watermelon mosaic virus 2 was predominant. The predominant cucurbit virus of 1981, WMV-1, was not detected in any of the 1985 samples.

Cucurbit viruses in New Jersy was detected by Davis and Muzuki, (1987). During 3 years of study, different viruses were associated with severe disease symptoms, depending on the years. In 1983 CMV caused the most severe disease in squash. Although watermelon mosaic virus 2 was the most prevalent. In 1984, the WMV structure of papaya ring spot virus caused destruction. ZYMV was detected for the first time in New Jersy in 1985 and caused severe losses in squash and other cucurbit crops. In field samples infected with various mixture of ZYMV, WMV-2 and PRSV-W, ZYMV usually predominated after rub inoculations of susceptible test plants and detections of ELISA. The assay of field samples was more reliable as an indications of the virus present in sample than ELISA of experimental test plants subinoculated with sap from field samples. ZYMV is highly aggressive and appears to have a competitive advantages over PRSV-W and WMV-2 in mixed infections. Most ZYMV isolates occurring in New Jersey were similar to the connecticut structure, however one isolate of ZYMV from squash designated ZYMV-N, unique from previously reported isolates in its ability to induce severe stunting and necrosis in squash, represents another biotype of ZYMV.

Yoshida and Iizuka (1987) reported watermelon mosaic virus II, ZYMV and CMV isolated from cucurbits in Hokkaido. Isolation, characterization and identification of these viruses from squash, bottle gourd and cucumber have confirmed their identity.

Akkawi *et al.*, (1987) reported control of mosaic disease affecting squash (*C.pepo*) in Jordan. In autumn 1982 mosaic incidence was reduced with intercropping with pepper (*Capsicum*), and maize. Pepper reduced the incidence of mosaic significantly at 34-48 days after transplanting. Intercropping with maize consistently reduced mosaic throughout the autumn growing season, but in spring (1983) under high inoculum pressure, incidence was lower only at 34, 48 and 62 days. The number of flying aphid caught in water traps was reduced when intercropping with maize was used.

Ghorbani (1988) reported isolation of ZYMV in the Tehran province, ZYMV caused systemic mosaic, distortion, vein banding and blistering of the leaves. The virus was identified on the basis of host range, particle morphology, serology and transmission characteristics.

Avgelis and Katis (1989) observed occurrence of SqMV in melons in Greece. SqMV was isolated from melons plants raised from imported seeds. Virus identification was based on host range, aphid and seed transmission tests, electron microscopy and serology. Seed testing by ELISA detected SqMV only in the cultivar Touralia imported from the USA.

A survey of virus infecting yellow summer squash by Sammon *et al.*, (1989) in South Carolina determined the incidence of CMV. papaya ring spot (type W) virus, watermelon mosaic virus II, squash mosaic virus and tobacco ring spot virus. Squash samples were collected during the summer and autumn of 1981 and 1982 in 7 countries where the commercial squash is grown. Virus detection methods included gel double diffusion and ELISA sample consisted of 2 or 3 leaves near the short apex. Some were collected from diseased plants and other were taken at random without regard for disease status. Viruses found (highest to lowest number of infected field) were WMV II, CMV, PRSWV and TRSV, SqMV was not detected. WMV II occurred in higher incidence than the other viruses with 100% infection found in 1 field in Greenville country. Random samples were not tested for Zucchini yellow mosaic virus but it was identified for the first time in South Carolina. ZYMV was purified and identified on the basis of host range, serology, morphology and aphid transmission. The symptomatology of these ZYMV isolates were similar to the connecticut biotype of this virus.

A new virus disease affecting cucurbits in Venezuela was reported by Herandez *et al.*, (1989) in which *C.moschata*, marrows, melons and watermelon cultivar Charslston Grey and Florida Gigante which were growing in experimental plots in Aragua and Flacon showed symptoms of virus infections. Papaya ring spot virus, potyvirus, watermelon

mosaic I potyvirus, squash mosaic comovirus and Zucchini yellow mosaic potyvirus (ZYMV) was identified from diseased plants. This is the first time ZYMV were identified from diseased plants in Venezuela and the first record of it in *C.moschata*. It was the next widespread virus in the plants studied and was transmitted in a non-persistent manner by *Aphis gossypii*.

Cucumber is infected with cucumber vein yellowing virus (CVYV), which is transmitted by whitefly and mechanical inoculation (Yilmaz *et al.*, 1989).

Squash mosaic virus in seed of melon (*Cucumis melo*) was reported by Franken *et al.*, (1990) by ELISA. An ELISA technique was developed in Netherland for the detection of squash mosaic virus (SqMV). In melon an antiserum was produced to a serotype-1 isolate from melon. The two ELISA variants investigator were an ELISA variants with simultaneous incubation of sample and an enzyme conjugate (ELISA 2). The sensitivity of ELISA was tested by mixing flour of ground infected and non-infected seeds in different proportions. SqMV was detected by both ELISA variants at dilution 1:160 (1 part of infected flour mixed with 159 parts of non-infected flour). However, ELISA-1 gave relatively higher absorbance values than ELISA 2 for nearly all dilutions. Since ELISA-1 is also faster than ELISA 2, ELISA 1 is advised for routine testing. In these tests, using sub samples of 100

melon seeds gave reliable detection of SqMV. ELISA 1 is now used in the Netherland for routine indexing of melon seed tests for SqMV.

A new Begomovirus infecting cucurbit formed viable reassortments with related virus in the Squash leaf curl virus cluster. It was designated, as cucurbit leaf curl virus which has sequence homology with cucurbit leaf crumple virus from California. The cucurbit leaf crumple virus DNA B shared highest nucleotide identity with Bean calico mosaic virus (BCaMV) (Brown *et al.*, 2002).

In cucurbits, Geminiviruses were reported to cause disease in bitter gourd, bottle gourd, cucumber, musk melon, pumpkin, ridge gourd, sponge gourd, squash, tinda and watermelon (Varma and Giri, 1998; Mansoor *et al.*, 2000; Singh *et al.*, 2001; Khan *et al.*, 2002; Revill *et al.*, 2003).

Muniyappa *et al.*, (2003) determined the host range of pumpkin yellow vein mosaic virus (PYVMV), from South India, which could infect *Nicotiana tobacum*, pumpkin, summer squash and winter squash only out of 19 species tested.

The virus causing leaf curl in squash in China has been characterized and the virus sequence was found to be closely related to Tomato yellow leaf curl Thailand virus (TYLCTHV). On the basis of these findings tentative name as squash leaf curl Yunnan virus (SLCYNV) was proposed by (Xie and Zhou, 2003).

A strain of SLCCNV from the Philippines has been characterized and has been designated as squash leaf curl China virus [Philippines] (SLCCNV-[PH] Kon *et al.*, 2003).

Verma *et al.*, (2004) reported Zucchini yellow mosaic virus in bottle gourd (*Lagenaria siceraria*) in India. The plant showed yellow mosaic symptoms. It showed 70% distribution in Pune, Maharashtra. It was the first report of ZYMV in bottle gourd from India.

Characterization of squash leaf curl virus and squash mild leaf curl virus was done by Brown *et al.*, (2005). They showed phylogeny and reassortment between Begomoviruses in SLCV clade. The nucleotide sequencing was done and sequences having accession number DQ 285016 for SMLCV and DQ 285014 for SLCV were submitted to Gene Bank.

Chapter -3
Materials & Methods

MATERIALS AND METHODS

1. MAINTENANCE OF VIRUS INOCULUMS:

1.1. Raising of test plants:

All the plants were grown in clay pots 4″ and 6″ diameter filled with a mixture of soil, sand and compost in a ratio of 2:1:1. The soil mixture was sterilized by autoclaving for one hour at a pressure of 20 lbs per sq inch. The clay pots were sterilized by rinsing in 4% formalin solution and the soil mixture autoclaved 24 hours earlier and sieved before use.

Seeds were sown in 12″ clay pots for raising seedlings except for plants belonging to cucurbitaceae and leguminosae which were raised singly by direct sowing in clay pots. Seedlings were transplanted singly in clay pots of 4″ and 6″ diameter at 2-3 leaf stage, when they were about 2 weeks old.

For inoculation the plants were used two weeks after transplantation. All the plants were kept in an insect proof glass house.

1.2. Virus Culture:

Young leaves of naturally infected plants of *Cucurbita pepo*. L showing mosaic symptoms were macerated using mortar and pestle with simultaneous addition of 0.01M phosphate buffer (pH 7.0). The macerate was filtered through double layered cheese cloth. Attempts of

single lesion inoculations were made to maintain a pure virus culture on suitable propagation host at 4-5 leaf stages. Inoculations were made by weekly mechanical inoculation onto health plants of propagation host to increase culture. Periodic checks were made on assay host, *Chenopodium amaranticolor* Coste and Reyn. to ensure biological purity of the virus.

1.3. Source of inoculum:

Young leaves of propagation host were used as a source of inoculum, prepared by macerating them in a mortar with pestle in 0.01M phosphate buffer (pH 7.0). For each gram of infected leaves 2 ml of buffer was used and the macerate was filtered through double layered cheese cloth. The sap thus obtained was used as standard inoculum (SI).

2. TRANSMISSION:

2.1 Mechanical Transmission: The fully expanded leaves of the plants to be inoculated will be dusted uniformly with carborundum 500 mesh as an abrasive and the standard inoculum will be applied gently but firmly on the upper surface of leaves with the help of fore-finger by keeping the other hand beneath the leaf to be inoculated. The inoculated leaves will be rinsed with gentle stream of water before the inoculum on the surface of leaves dried up. If the rate of transmission is not promising, some chemical will be mixed with the inoculum so as to enhance the rate of transmission. Additive in the inoculum will include

sodium sulfite, 2-mercapto-ethanol, ethylene diamine-tetra-acetic acid, sodium diethyl-dithiocarbonate and thioglycolic acid either alone or in various possible combinations if needed.

2.2. Biological Transmission: Attempts will be made to find out the vectors of virus in the field, transmission by insects, soil, dodder (*Cuscuta spp.*), seeds, grafts and pollen will be studied.

2.2.1. Insect Transmission:

(a) Transmission by aphids:

Adult aphids found transmitting the disease during preliminary investigations were used to study virus vector relationship (non-persistent, semi-persistent or persistent).

(b) Raising of virus free aphids:

Viviparous adults were starved for 2,4,6 and 8h at room temperature in a petridish and then placed upon a detached leaf of an appropriate healthy host in a petridish. The atmosphere inside the petridish was made humid by covering the inner surface of petridish with wet filter paper. Newly borne nymphs were transferred to a fresh and healthy test plant. The aphid colonies thus developed were used as healthy colonies of virus free aphids. The aphids from one plant to other were transferred with the help of moistened tip of camel's hair brush type A, No.1. Colonies of virus free aphids were raised on suitable host plant in cages having wooden frames. The top and two sides of cage

were closed by glass and the remaining sides were closed by wire gauze. A fluorescent tube was fixed in the cage to keep the aphids under long day conditions to get the apterous (wingless) aphids. The plants were kept on a zinc-tray and the bottom of the tray was covered with a layer of moist sand to prevent the passing of the aphids through chinks between the tray and the rim of the cage.

(c) Mode of Transmission:

To established the mode of transmission following procedure was adopted;

NON PERSISTENT:

Pre-acquisition starvation periods	–	1 to 2h
Acquisition access period	–	2 to 5 min
Inoculation access period	–	24 h
Number of aphids/ plant	–	10

The nymphs were starved for 1 to 2h in a petridish having the inner surface covered with a wet piece of filter paper before an acquisition access period of 2 to 5min. on the leaf of the diseased plant. After allowing acquisition feeding time, the nymphs in batches of 10 were transferred to each healthy seedling and the plants were covered with Leztz cages for an inoculation access period of 24h, the nymphs after the end of inoculation access were killed by spraying 0.02 percent cypermethrine (insecticide) and the plants were kept in an insect proof

glasshouse for the development of symptoms. Back inoculations for each plant were made to an appropriate local lesion host, *C. amaranticolor*.

PERSISTENT

Acquisition access period	–	24h
Inoculation access period	–	48h
Number of Aphids/plants	–	10

The virus free aphids, without subjecting them to starvation were allowed 24h acquisition feeding time on diseased leaves. After the completion of acquisition feeding, 10 aphids were transferred to each test plant where they were given an inoculation feeding period of 48 h. Aphids were killed by spraying an insecticide cypermethrine (0.02% solution). The test plants were kept in an insect proof glass house to observe the development of symptoms. Back inoculations from the plants on which aphids were given inoculation feedings were made on a local lesion host *C. amaranticolor*.

2.2.2. Transmission by White Flies:

(a) Source of Virus free white-flies:

White flies (*Bemisia tabaci* Genn) collected from field were caged on a healthy plant of *N.glutinosa* for egg laying. After 10 days the adults were removed from the cage. New born white fly adults developing after 7-8 days were allowed further multiplication. Insect colonies so raised were virus free and used for transmission studies.

(b) Handling of white-flies:

The method described by Rathi and Nene (1974) was used for handling of white flies.

(c) Transmission:

Non-viruliferous white flies were allowed acquisition and inoculation access period of 24h each on diseased and healthy plants respectively. Cypermethrine (0.02%) was sprayed to kill the white flies after inoculation. The test plants were kept for observation of symptoms.

2.2.3. Graft Transmission:

Attempts will be made for side wedge grafting. Infected scions will be grafted on healthy stock and kept under appropriate light and humidity condition to allow successful union which is necessary for transmission.

2.2.4. Dodder Transmission:

Seeds of dodder (*Cuscuta reflexa* Roxb. and *C. chinensis* Lam.) were germinated on moist filter paper placed in petri-dish and then transferred in 4" clay pots, sterilized with formaline (4%) and containing sterilized soil mixture. When the dodder plants were about 6" long, they were trained on a suitable host plant susceptible to the virus and the host plants (on which the dodder was trained) were inoculated after one week. When the dodder had been established on inoculated plants, a healthy test plant in another pot was placed near the pot (having

inoculated plant with dodder established on it) and the tips of the branches of dodder were detached, placed in the axil of the healthy test plant and allowed to establish there. The plants were left as such for about 2 months to develop the symptoms. Back inoculations were made on *C. amaranticolor* to confirm the presence of virus.

2.2.5. Soil Transmission:

Soil around the naturally infected plants were collected from the field and sieved to remove roots and debris etc. such soil was divided in to two parts. One part was filled in a gunny bag and was autoclaved at 15 lbs/ $inch^2$ for one hour and the other part of soil was left as such and was filled in pots. Healthy seedlings were sown in pots containing sterilized and unsertilized soil. Plants of both the sets were kept for observation of symptoms in an insect proof glass house. The presence of virus was confirmed by making back inoculations on the test plants.

2.2.6. Seed Transmission:

To determine seed transmission of virus, experiments were carried out as follows:

(a) Sowing method:

Seeds were collected from infected and healthy plants and were sown in autoclaved soil in an insect proof glass house. After seedling emergence, the plants were observed till 5-6 weeks and were sprayed

with 0.02% cypermethrine at weekly intervals to prevent insect infestation.

(b) Infectivity test method:

Leaf tissues of such plants (grown as above) were macerated in 0.01M phosphate buffer (pH 7.0) and then sap obtained was inoculated manually on local lesion host, *C.amaranticolor* to ascertain the presence of virus in them.

3. HOST RANGE STUDIES:

Several species of plants, belonging to different families will be screened for the susceptibility to the virus causing mosaic disease on squash. Standard inoculum was used for inoculation of all plants. At a time at least 5 plants of each species were inoculated and the same number kept as a control. Plants at 5-6 leaf stage were used and all the fully expanded leaves were inoculated. The inoculated plants were observed upto two months for the development of symptoms. The time sequence and severity of the symptoms were noted. Inoculated plants exhibiting no symptoms were kept for about 8 weeks for observation. Back inoculation were made to a test plants from all the inoculated plants.

4. VIRUS-VECTOR RELATIONSHIPS:

In order to determine the relationship between virus and the vector, the method would depend on the type of the vector group

involved in transmission. However, in general the variabilities including number of insects per plant, different pre-acquisition starvation periods, varying acquisition and inoculation access periods will be worked out along with effect of moulting of insect on various retention and latent periods in the vector.

5. EFFECT OF DIFFERENT BUFFERS ON THE INFECTIVITY:

Various buffers (phosphate, borate, citrate and acetate) at different pH and molarities will be used and tested to find out the most suitable one in which virus infectivity is retained maximum. Young infected leaves will be macerated in a mortar with pestle using a buffer (any of the above mentioned) as extraction medium. The sap obtained after filtrating it through double layered cheese cloth will be inoculated on the leaves of local lesion host following the usual method of inoculation. All buffers will be tested in the same way, and a buffer at a pH and molarity in which virus infectivity is higher will be selected and used regularly as an extraction medium for the virus being used.

6. VIRUS CONCENTRATION IN DIFFERENT PART OF THE HOSTS:

To determine the virus concentration in different parts of the host plant, 10-15 days earlier inoculated plants will be uprooted carefully and washed. The plants will be blotter dried. Root, stem and leaf tissue will be cut separately into pieces. Equal amount of root, stem and leaf tissue will be macerated separately in mortar and pestle using a suitable buffer.

Sap obtained from each sample will be inoculated separately on a local lesion host using usual method of inoculations.

7. SELECTION OF SUITABLE PROPAGATION HOST AND AN ASSAY HOST:

To find out a suitable propagation host several plants, susceptible to the virus will be inoculated and that showing most prominent symptoms will be selected. A plant exhibiting following characters will be selected.

i) Rapid seed germination and fast growth.

ii) Short incubation period of the virus.

iii) Peak concentration of virus within the short period after inoculations.

iv) Absence of virus inhibitors.

v) More yield of infected tissue with good virus concentration.

Assay of virus will be carried on a local lesion host. To search out a local lesion host several commonly used plants will be tested. However, in case of non-availability of a local lesion host, assay tests of virus will be carried out on a systemic host.

8. BIOPHYSICAL PROPERTIES:

To determine the dilution end point, thermal inactivation point and longevity *in vitro*, methods described by Noordam (1973) will be employed.

8.1 Dilution end point (DEP):

By adding suitable buffer, ten fold dilutions (10^{-1}, 10^{-2}.....10^{-6}) will be made of the sap obtained from infected leaves of the propagation host after homogenizing them in a mortar with pestle. Each sample will be inoculated on the leaves of the local lesion host following the usual method of sap inoculation. In this way the dilution at which virus loses its infectivity will be determined.

8.2. Longivity *in vitro* (LIV):

In Sap: The infected leaves of the propagation host will be homogenized in a mortar with pestle while using a suitable buffer and the homogenate will be filtered through two layers of cheese cloth and the sap thus obtained, will be divided into two parts one part will be kept at room temperature (25 ± 5°C) and the other part will be kept at 4°C of temperature. After every 6h interval, a small amount of the sap from both parts will be taken and inoculated on the leaves of local lesion host following the usual method of sap inoculation. In this way the time after which the virus loses its infectivity will be recorded.

8.3. Thermal inactivation point (TIP):

The sap obtained by the same method as mentioned above, will be divided into 8 aliquots of 5 ml each and kept in glass vials. The glass vials will be held in a water bath in such a way that the sap level in the vial is below the water in bath. The different aliquots will be heated at

40, 45, 50..... 70, 75°C, for ten minutes and cooled under running tap water, immediately after heating. Each heated aliquot will be inoculated on the leaves of a local lesion host. One aliquot left at room temperature will also be inoculated and will serve as control.

9. EFFECT OF VARIOUS ADDITIVES ON VIRUS INFECTIVITY:

To work out whether the stability and infectivity of the virus will get increased, several additives (Sodium sulfite, DIECA, EDTA, sodium thioglycollate, mercapto ethanol) will be used. In case, the infectivity get enhanced, the most suitable additive will be selected and routinely added to the medium for virus extraction.

10. PURIFICATION:

After selecting a suitable buffer, a propagation host(s), an assay host(s) and biophysical properties, attempts will be made to purify the virus under consideration.

The infected leaves of the propagation host will be macerated by usual and suitable method and the macerate will be passed through a double layered cheese cloth. The sap thus obtained will be given a low speed centrifugation at 5000g for 10 minutes. The supernatant (sap) will be subjected to various clarification procedures.

10.1 (a) Organic solvent:

Organic solvents (butanol, ethylalcohol, chloroform, carbon tetrachloride and di-ethyl ether) either separately or in combination

(such as chloroform butanol) will be used in two ways for the removal of the extraneous plant material from the infected tissue.

i) By macerating the infected tissue by using a mixture of suitable buffer and organic solvents, or

ii) By adding requisite amount of solvent in crude sap obtained after macerating the infected tissue in buffer and filtering through two layers of cheese cloth.

The mixture will be incubated for 30 minutes and then centrifuged at 5,000g for 15 minutes. The aqueous layer will be separated. The effect of solvent on the virus infectivity will be tested by assaying the aqueous layer for active virus content on a local lesion host.

(b) Silver nitrate:

Different volumes of 1 percent silver nitrate solution will be added drop by drop to the standard inocolum (1/5) and stirred simultaneously. The mixture will be left at room temperature for 30 minutes and thereafter, centrifuged at 5000g for 15 minutes. The supernatent thus obtained will be bioassayed an local lesion host for virus infectivity.

Out of clarification methods described above, one will be standardized and used as clarification methods in the clarification of the virus being studied.

10.2 Concentration of Virus:

The sap obtained after clarification treatment as described above will be used for concentration of virus by any of the following methods,

a) Differential Centrifugation:

The ultra centrifugation will be worked out in model L7-60. Beckman preparative ultracentrifuge using rotor T-60. Normally high speed centrifugation will be done at 50,000g unless otherwise stated. The pellet, thus obtained will be dissolved in suitable buffer. Low speed centrifugation will be performed at 10,000 in a Remi T-24 centrifuge or any other same type of centrifuge. The number of cycles and the time of centrifugation at different rpm will be carried out keeping in view the stability of the virus and it sedimentation. Activity of different samples in supernatant and the pellet will be assayed on local lesion host.

b) Precipitation:

i) Polyethylene glycol (PEG)

Polyethylene glycol (Mol. wt 6,000) will be used for precipitating the virus in clarified sap. Precipitation of the virus will be tried with 2,4,6,8,10 and 12 percent PEG separately. In every case, the variation in salt (NaCl) concentration and its impact as precipitation of the virus will be standardized. After the addition of requisite quantity of PEG and NaCl to the clarified sap, the mixture will be stirred on a magnetic stirrer till both (PEG and NaCl) are dissolved completely.

ii) Ammonium sulphate:

Different quantities (10-40%) of ammonium sulphate $(NH_4)_2 SO_4$ (w/v) will be added to clarified sap (1/1). The mixture will be stirred at 8± 2°c in an ice bucket till the $(NH_4)_2 So_4$ crystal are dissolved completely. The mixture will be incubated at 4± 1°c for 2 hours and centrifuged at 5,000g for 15 min, to collect the precipitate.

The pellet thus obtained by PEG and $(NH_4)_2 So_4$ precipitation will be dissolved separately in a suitable buffer and re-centrifuged at 5,000g for 5 minutes. Supernatant thus obtained will be assayed on local lesion host.

11. FURTHER PURIFICATION BY DENSITY GRADIENT CENTRIFUGATION:

Concentrated virus samples obtained by the methods described above will be subjected to further purification using density gradient centrifugation (Brakke, 1951, 1960).

Linear sucrose gradient columns will be prepared by layering 7,7,7, and 4ml of 0.01m phosphate buffer (pH 7.0) having 400, 300,200 and 100mg sucrose per ml, respectively in a 1x3" tube. The solutions of different concentrations will be layered using a pipette with a broad orifice.

The heaviest solution will be layered first and the solutions of decreasing concentration will be layered on the top of each other. The

column will be used after standing for 24h in a refrigerator. 2ml of the virus preparation will be floated on the top of column and the column will be centrifuged immediately after floating the virus preparation to avoid droplet sedimentation. The column will be centrifuged in SW-25.1 rotor in L7-60 preparative ultracentrifuge. The acceleration upto a few hundred rpm will be made gradually. The tubes will be centrifuged for 3½ hours. After centrifugation the tubes will be examined in a dark room by projecting a beam of light down the tube from the top. The virus zone scattering the light will be removed from the tube by 20 gauze 10 cm long needle bent twice at right angles attached to a hypodermic syringe.

12. UV-SPECTROPHOTOMETRY:

The virus preparation will be examined in Beckman DU-7 model ultraviolet absorption spectrophotometer to evaluate the different methods of purification and to a certain the purity of virus isolated.

Ultraviolet radiations are absorbed in a characterize manner by the virus (nucleo-protein) containing solutions. Absorbance of samples will be studied in UV-range (230-320nm) and graphs will be plotted. Values at A max/min $A_{280/260}$ and $A_{260/280}$ will be calculated to know the opproximate percentage of nucleic acid.

13. ELECTRON MICROSCOPY:

Shape and size of virus particles will be studies in electron microscope.

13.1 Leaf dip method:

Method described by Brandes (1964) will be followed for leaf dip preparations. One drop each of 20% potassium phosphotungstic acid (PTA) and uranyl acetate will be placed separately on several formvar coated copper grids having carbon backing. The freshly cut ends of infected leaves will be dipped in the drop for 2-4 seconds. Such grid will be allowed to dry for sometime and thereafter examined under electron microscope at various magnifications.

13.2 Procedure with purified virus preparation:

A small droplet of purified virus preparation will be placed on farmvar coated copper grids having carbon backing, then a small drop of suitable strain (either PTA or uranyl acetate) will be added to the virus suspension. The excess fluid will be absorbed with a small piece of filter paper leaving a very thin film of fluid on the grids, which will be dried at room temperature. Such grids will be examined under electron microscope.

14. SEROLOGY:

Specific antigen and antibody reaction is one of the useful techniques either for assigning the virus to a particular group or to

differentiate it at the strain level. Antisera to the virus under consideration will be prepared for the identification of the virus as well as for testing the latent infection in certain hosts. Besides it would also be useful for ascertaining the seed transmissible nature of the virus through routine serological methods or by enzyme linked immuno-sorbent assay (ELISA).

14.1 Raising of Antisera:

Young healthy rabbits, approximately 3lbs in weight will be used for production of antisera. The purified or partially purified virus preparation will be used as antigen.

To work-out the effect of injection on the formation of antibodies as well as titre of the antiserum, antigen (virus preparation) will be injected intravenously or intramuscularly or in both ways.

The antigens will be administered intravenously through the marginal ear vein of the rabbit using a clinical syringe with a fine needle. Five to seven weekly injection of virus preparation of 2ml. each will be administered intravenously through the marginal vein of the ear. For intramuscular injection, antigens will be emulsified with an equal volume of Freund's incomplete adjuvant. The injections of the virus adjuvant mixture of 3ml each at an interval of 2 weeks will be administered intramuscularly in thigh of the same rabbit which has been given intravenous injections of antigens.

Test bleedings will be made several times from the ear of the rabbit at different intervals after the administration of last intramuscular injection to check the antibody titre in serum. After the titre has reached its maximum, the immunized rabbits will be finally bled by giving a sharp incision an the marginal vein of the ear, which has not been used for injecting the antigens. About 10-15 ml of the blood will be collected and allowed to clot at room temperature (20±5°c) for 2 hours and kept overnight in a refrigerator. Serum containing antibodies (antiserum) will there after be separated and centrifuged at 1,000 rpm for 5 minutes to remove fibron, blood cells etc. the straw yellow colored antiserum will be collected and stored for serological studies.

14.2 ELISA (Enzyme Linked Immuno Sorbent Assay):

Direct antigen coating ELISA (DAC-ELISA) will be performed using virus antisera. The basic technique will be the same as described by Clark and Adams (1977). It is used for assaying by either a competitive or a double antibody methods and for assaying a specific antibody (Ab) by an indirect method. Here through this methods we measure Ab levels.

The putative antiserum will be reacted with specific Ag attached to a solid phase. Only specific antibody (Ab) molecule bind to the antigen (Ag) and all other material will be washed away. Exposure of this complex to enzyme labelled anti-IgAb results in binding of specific

antibody molecule adsorbed from the original serum. The complex will be washed and the substrate of the enzyme will be added, resulting in the activity proportional to the amount of specific antibody in the original serum. Few enzymes like Horse radish-per-oxidase (HRPO) or alkaline phosphatase have been used to label antibodies.

PROCEDURE:

1. First 100µl of antigen (1µg) in carbonate buffer pH 9.6 will be coated in each well of micro ELISA plates.
2. Plates will be incubated at 37°C overnight in a humid chamber.
3. Wells will be blocked by 100µg of 1% BSA or non-fat dried milk (5%) in carbonate bicarbonate buffer in each well for 6 hrs at 37°C in humid chamber.
4. Wells will be washed with Tween-TBS and incubate 100µl of antiserum in each well for 6hrs.
5. Wells will be washed extensively with Tween-TBS to remove unbound antigens (virion particles) and then 100µl of antisera enzyme conjugate will be added to each well.
6. Incubate plate for 1h at 37°C.
7. Wells will be washed with Tween-TBS and 100µl of substrate. Then we wait till the colour develops.

Lastly, absorbance will be measured at 450nm in ELISA reader. (Bio Med, USA)

15. ISOLATION OF NUCLEIC ACID:

Phenol detergent method will be used to isolate the nucleic acid of viruses. To a 2.5 ml of purified virus preparation, 0.05ml of 6% sodium dodecyl sulphate and 2.5ml of water saturated phenol will be added. The phenol used will be redistilled and stored at 10°C after adding distilled water. The mixture will be stirred in a glass tube on a magnetic stirrer for 10 minutes and then centrifuged for 5 minutes at 3,000 rpm in a clinical centrifuge.

The mixture will be separated into two layers, the upper aqueous layer and the lower phenol layer containing sodium dodecyl sulphate. The top aqueous layer will be drawn with a pipette. To the lower phase 2.5ml of 0.01M phosphate buffer (pH 7.0) will be added and stirred for 10 minutes and then centrifuged for 5 minutes at 3,000 rpm. The aqueous phase will be drawn off and polled together with aqueous phase obtained at previous step and stirred for 10 minutes with an equal volume of phenol followed by centrifugation. The aqueous phase will be extracted once more with half the volume of phenol. Traces of phenol will be removed from the aqueous phase by extraction with ether. The nucleic acid will be precipitated by the addition of 2ml of ice cold ethanol to the solution. The precipitate will be polluted out by centrifugation for 15 minutes at 7,500rpm. The pellet will be suspended in 0.01M phosphate buffer (pH 7.0) and centrifuged for 15 minutes at 10,000 rpm to remove any insoluble material present in the

precipitation, and the supernatant thus obtained will be tested for infectivity and type of the nucleic acid (RNA or DNA).

15.1 Infectivity of viral nucleic acid:

The infectivity of viral nucleic acid will be assessed by inoculating the nucleic acid preparation on local lesion host. Several dilutions of nucleic acid preparation will be made and inoculated on the local lesion host and the number of local lesions developed will be compared with the corresponding dilutions of the virus preparation.

15.2 Type of nucleic acid:

It is well known that RNA and DNA differ in their chemical composition with respect to base and sugar involved in their composition. RNAs are known to contain ribose sugar and uracil base (other three bases being adenine, cytosine and guanine) while DNAs contain thymine (other three bases are same as in case of RNA) and deoxyribose sugar.

Thus, test will be performed to study the type of sugar. Diphenylamine test for deoxyribose or orcinal test for ribose sugar will be used for ascertaining the type of nucleic acid in virus under investigation.

16. STUDIES ON PROTEIN OF THE VIRIONS:

Attempts will be made, while using standard methods to determine the approximate molecular weight of proteins associated with the virions.

Chapter -4
Results

RESULTS

1. NATURAL SYMPTOMS:

Naturally infected plants of squash (*Cucurbita pepo* L.) showed mosaic symptoms on leaves. At advanced stage of infection, the infected plants showed vein clearing, reduction in leaf size with retarded vegetative growth of plant (Fig.1).

2. TRANSMISSION:

By Sap:

The virus causing mosaic disease on squash (*C. pepo* L.) was readily transmitted by sap extracted in 0.01 M phosphate buffer (pH 7.0) from Squash to Squash, *Nicotiana* species and other susceptible hosts. The transmission of the disease, using carborundum (500 mesh) as an abrasive, was 90-100% by sap. In subsequent studies, the infected *Nicotiana* leaves were macerated in 0.01M phosphate buffer pH-7.0 (1:2 w/v) and the sap thus obtained was used as standard inoculum (SI).

3. HOST RANGE AND SYMPTOMATOLOGY:

To determine the host range of virus causing mosaic disease on squash various species and cultivars of plant belonging to different families were inoculated mechanically with standard inoculum. To ascertain the presence or absence of the virus, back inoculations from all inoculated plants were made on *C. amaranticolor* and the plants

inoculated developed symptoms as described below and the virus was recovered on back inoculation in most of the cases.

Chenopodiaceae:

Chenopodium amaranticolor Coste and Reyn.

Pin head type of local lesions were formed after 5-6 days of inoculation. Initially there were chlorotic local lesions which later on turned into necrotic local lesion. (Fig.3).

Cucurbitaceae:

Cucurbita pepo L.

Systemic symptoms appeared after 14-15 days of inoculation on the new emerging leaves in the form of vein yellowing, mosaic and mottling. In severe condition the leaves became deformed and reduced in size. (Fig. 1&2).

Cucumis sativus L.

Systemic symptoms appeared after 14-15 days of inoculation in the form of severe mosaic followed by raised green areas and mottling (Fig. 4&5).

Solanaceae:

Datura metel L.

Necrotic local lesions appeared after 5-6 days of inoculation. However, in later stage mosaic mottling, leaf deformation and reduction in size of leaves was also observed (Fig. 6 & 7).

Nicotiana tabacum cv. Anand 2 Bidi type

Systemic infection were observed after 12-13 days of inoculation in the form of mild mosaic (Fig.8).

Nicotiana tabacum cv. Harison's Special

Necrotic local lesions were observed on the inoculated leaves after 5-7 days of inoculation (Fig.9).

Petunia hybrida

Necrotic local lesions developed on the inoculated leaves after 5-7 days of inoculation (Fig.10).

Susceptible host plants	**Symptoms**
Chenopodiaceae:	
Beta vulgaris L.	NLL
Chenopodium album L.	NLL
C. amaranticolor Coste and Reyn.	NLL
Cucurbitaceae:	
Cucumis melo L.	Mm, Vc, Cp
C. sativus L.	SM, Mm
Cucurbita pepo L.	Mm, Vc, S
C. moschata Dusch.	Vc, Mm
C. maxima.	Vc, Mos, Ld, S
Lagenaria siceraria Standl.	Vc, CLL
Luffa cylindrica Roem.	Vy, Mos
L. acutangula Roxb.	CLL, Mos

Solanaceae:

Datura metal L.	NLL, CLL
D. stramonium L.	NLL, CLL
Nicotiana tabacum cv. Samsun NN.	Vy, Mos
N. tabacum cv. Anand 2 Bidi type.	MM, NLL
N. tabacum cv. Harison's Special.	NLL
N. glutinosa.	Vc, Mos, S
Lycopersicon esculantum L.	Ld, Mm, Wt
Solanum melongena.	Vy, S, Vn
Petunia hybrida.	Vy, S

Leguminosae:

Pisum sativum L.	N, Wt
Trigonella foenum graceum L.	NLL
Vicia faba L.	Mm

CLL	= Chlorotic local lesion	Cp	= Chlorotic patches
Ld	= Leaf Deformation	Mm	= Mosaic mottling
MM	= Mild mosaic	Mos	= Mosaic
N	= Necrosis	NLL	= Necrotic local lesion
S	= Stunting	SM	= Severe Mosaic
Vc	= Vein clearing	Vn	= Vein necrosis
Vy	= Vein Yellowing	Wt.	= Wilting.

Fig.1 Infected leaf of *Cucurbita peop* L. showing disease symptoms in the form of vein clearing & mild mosaic.

Fig.2 Infected leaf of *Cucurbita peop* L. showing disease symptoms in the form of mosaic mottling.

Fig.3 Infected leaf of *Chenopodium amaranticolor* Coste and Reyn. showing infection in the form of chlorotic local lesions.

Fig.4 Infected leaf of *Cucumis sativus* L. showing infection in the form of mosaic mottling.

Fig.5 Infected leaf of *Cucumis sativus* L. showing infection in the form of severe mosaic.

Fig.6 Infected leaf of *Datura metal* L. showing infection in the form of necrotic local lesions.

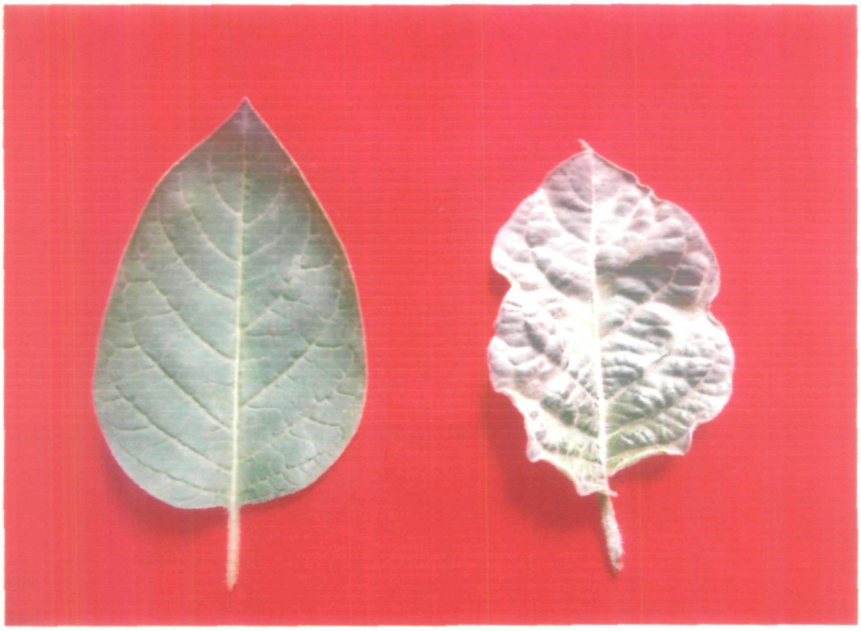


Fig.7 Infected leaf of *Datura metal* L. showing infection in the form of mosaic mottling (right) and healthy leaf (left).

Fig. 8 Infected leaf of *Nicotiana tabacum* cv. Anand 2 Bidi type showing disease symptoms in the form of necrotic local lesion & mild mosaic.

Fig. 9 Infected leaf of *Nicotiana tabacum* cv. Harison's special showing disease symptoms in the form of necrotic local lesions.

Fig. 10 Infected leaves of *Petunia hybrida* showing infection in the form of necrotic local lesions.

Non Host

No symptoms (local/systemic) were produced and no virus could be recovered on back inoculation to *C. amaranticolor* from the following plant species and cultivars. These plants were kept under observation for 6 weeks after inoculation.

Amaranthaceae:

Amaranthus caudatus L.

Apiaceae:

Coriandrum sativum L.

Asteraceae:

Tagetus erecta L.

Calendula officinalis L.

Ageratum maxicanum Sims. cv. Blue Mink.

Helianthus annus L.

Zinnia elegans Jacq. cv. Suttons Giant Double Mixed.

Brassicaceae:

Brassica compestris L.

B. oleracea L. var. botrytis cv. Snow Ball.

B. oleracea L. var. botrytis cv. Pusi.

B. rapa L. cv. Purple Top White.

Raphanus sativus L. cv. Bombay Red.

R. sativus L. cv. Chinese Pink.

R. sativus L. cv. Pusa Himani.

Chenopodiaceae:

Chenopodium murale L.

C. quinoa L.

Cucurbitaceae:

Citrullus vulgaris Schrad var. Fistulosus cv. Dilpasand.

Momordica charantia L. cv. Poona Long Green.

M. charantia L. cv. Coimbatore Long.

Liliaceae:

Alium cepa L. cv. Red Globe.

Malvaceae:

Abelsmoschus esculentus (L.) Moench cv. Pusa Sawani.

Solanaceae:

Capsicum annuum L. cv Cluster Suryamukhi.

Nicotiana tabacum L. cv. CTRI Special type FCV.

N. tabacum L. cv. Jayasri type FCV.

N. debneyi Domin.

N. longiflora Cav.

N. occidentalis Wheeler.

N. plumbaginifolia Viv.

S. nigrum L.

Verbenaceae:

Verbena hybrida L. cv. Tall Mixed.

4. SELECTION OF LOCAL LESION HOST:

Three local lesion host of the virus causing mosaic disease of squash viz. *Chenopodium amaranticolor* Coste and Reyn, *C album, N.tabacum* cv-Harison's Special were compared to select the most suitable one. The inoculum prepared from infected *N. tabacum* was inoculated to the above host and local lesion were counted 3-4 days after inoculation.

Table 3: Comparative study of different local lesion host of virus causing mosaic disease on squash.

Local lesion host	No. local lesion/leaf*
Chenopodium amaranticolor	28
C. album	21
Nicotiana tabacum cv. Harison's Special	25

* Average number of local lesions/plant based on three experiments with three plants having 6 leaves each.

The lesion produced on *C. amaranticolor* were necrotic and were easily countable and consistent. On the basis of these characteristics *C. amaranticolor* was choosen as the assay host and test plant for the present virus.

5. PROPERTIES OF THE VIRUS IN PLANT SAP:

Parameters such as thermal inactivation point (TIP), dilution end point (DEP) and Longevity *in vitro* (LIV) in the identification of plant viruses provide information about the best environment in which to keep the virus and maintain its infectivity. Although, these studies have

restricted value (Ross-1964) but are of great help in determining the procedure for purification of the virus and in its characterization. To study these properties, experiments were carried out using *Nicotiana tabacum* as donor host and *C. amaranticolor* as assay host of the virus. Three experiments of each property were performed using *C. amaranticolor* as an assay host.

a) Thermal inactivation point (TIP)

The virus in crude sap was found to be infectious after being heated for 10 min. at 60°C but was found to be inactive after being heated at 65°C. (Table 4). Thus, the thermal inactivation point (TIP) of the virus lies between 60°C to 65°C.

Table 4: Effect of Temperature on the infectivity of the virus.

Temperature °C	Average no. of local lesions /leaf*
40	49
45	34
50	23
55	13
60	6
65	0
70	0
75	0

* Average number of local lesions/plant based on three experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

b) Dilution end point (DEP)

The virus in crude sap was found to be infectious at a dilution of 10^{-4} but no local lesion were observed when the sap was diluted to 10^{-5} (Table 5). Therefore, the dilution end point of virus lies between 10^{-4} and 10^{-5}.

Table 5: Effect of dilution of crude sap on the infectivity of the virus.

Dilution	Average no. of local lesions /leaf*
Undiluted	42
10^{-1}	31
10^{-2}	18
10^{-3}	11
10^{-4}	5
10^{-5}	0
10^{-6}	0

* Average number of local lesions/plant based on three experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

c) Longevity *in vitro* (LIV)

The crude sap from a propagation host was obtained and divided into two parts. One was kept at room temperature (25+5°C) and the other in a refrigerator (4°C). Each sample was assayed separately on *C. amaranticolor* after a specific period of storage (Table 6&7). The virus retained infectivity in expressed sap upto 60h (20± 5°C) and loose its infectivity thereafter where as at 4°C the virus retained its infectivity upto 3 weeks.

Table 6: Longevity *in vitro* of crude sap when stored at room temperature (20 ± 5°C).

Storage in hours	Average no. of local lesions /leaf*
Immediately after extraction	
After 6 hours	65
12	54
18	42
24	34
30	31
36	30
42	22
48	17
54	11
60	5
66	2
72	0
78	0

Table 7: Longevity *in vitro* of crude sap when stored at 04°C.

Duration	Average no. of local lesions /leaf*
Immediately after extraction	68
After 1 week	48
After 2 week	24
After 3 week	5
After 4 week	0
After 5 week	0

* Average number of local lesions/plant based on three experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

6. EFFECT OF BUFFERS:

Effect of acetate, boric acid borax, citrate, citrate phosphate and potassium phosphate buffers at different pH values were compared for extraction of virus causing mosaic disease in squash from infected leaves of *N. tabacum* plants.

Table8: Effect of various buffers at different pH levels.

Buffer	pH	No. of local lesions/leaf*
0.1M Acetate buffer	4.5	4
	5.0	11
	5.5	**15**
	6.0	10
0.1 M Citrate buffer	4.0	5
	4.5	7
	5.0	18
	5.5	20
	6.0	25
	6.5	**26**
0.1 M Borate buffer	7.5	18
	8.0	**34**
	8.5	33
	9.0	28
0.1 M Phosphate buffer	4.5	11
	5.0	18
	5.5	28
	6.0	36
	6.5	44
	7.0	**48**
	7.5	43
	8.0	30
	8.5	10
	9.0	5

* Average number of local lesions/plant based on three experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

Results presented in the Table 8 indicated that the maximum infectivity was obtained with potassium phosphate buffer (pH 7.0).

7. EFFECT OF MOLARITY:

Attempts were made to determine the most suitable molarity (ionic strength) of potassium phosphate buffer (pH 7.0) for virus infectivity. At different molarities the virus infected leaves of *N. tabacum* were macerated in phosphate buffer (pH 7.0) separately. The samples were assayed on local lesion host, *C. amaranticolor* to compare virus infectivity.

Table 9: Effect of different molarity on the infectivity of virus using phosphate buffers (pH 7.0).

Molarity	No. of local lesions/leaf*	Relative infectivity (% age)
0.001M	45	75.00
0.05M	54	90.00
0.01M	60	100
0.1M	52	86.66
0.5M	50	83.33
1.0M	38	63.33

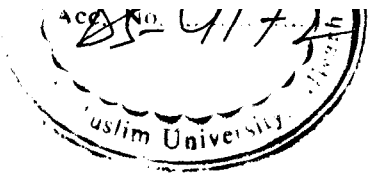
* Average number of local lesions/plant based on three experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

It is clear from the Table 9 that the extraction of infected leaves in 0.01M potassium phosphate buffer at pH 7.0 gave the highest infectivity.

Thus for the extraction of virus from infected tissues and maintaining the extracts at different stages of purification, 0.01M potassium phosphate buffer (pH 7.0) was found to be suitable at which virus infectivity was highest.

Chapter -5
Discussion

DISCUSSION



The virus causing mosaic disease on squash (*Cucurbita pepo* L.) was observed in the month of April (2006) in the fields of village Panjipur, Aligarh. The symptoms observed were in the form of mosaic disease on leaves. The infected plants were comparatively shorter and growth was retarded. The culture of the virus causing mosaic disease was maintained in an insect proof glass-house by a single lesion inoculation on to *Nicotiana tabacum* cv. Harrison's Special as well as on Squash.

From the pure culture obtained by a single lesion inoculation all the preliminary studies were carried out e.g. biophysical properties, host range studies and effect of various buffers with different molarity and pH.

The comparison of the host range, symptomatology and biophysical properties of the virus, with other viruses reported to infect squash (*C.pepo* L.) revealed that the present isolate differs markedly from them. The virus under study could not be compared with the viruses already described in the literature due to lack of sufficient information. However, the present isolate also differ in its host reactions and physical properties from the virus described by Anderson (1954). The present strain is totally dissimilar with the seed borne mosaic virus of muskmelon described by Radar *et al* (1957) but show some similarity

with the melon and squash mosaic virus (Lindberg *et al.*, 1956) in its mode of transmission and thermal death point. The squash mosaic virus infecting squash (*C.pepo* L.) described by Freitag (1941, 1956) differs in its host range, physical properties as well as mode of transmission. The mosaic disease of squash (*C.pepo* and *C. maxima*) described by Kamuro (1956, 1957) and pumpkin described by Shankar *et al.*, (1972) also differ as their host range is restricted to the family cucurbitaceae only which is contrary to the behaviour of CMV which can also infect some members of chenopodiaceae, leguminosae and solanaceae. The virus however resembles to CMV of Smith (1957) in its symptoms, host range, mode of transmission and physical properties.

From the information obtained on host range, biophysical properties and sap transmission of the virus studied herein, nothing can be said about the identity of the isolate obtained from the *C. pepo* which requires detailed studies e.g. purification, electron microscopy, serological tests and molecular diagnosis.

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